(19) Europäisches Patentamt
European Patent Office
Office europeen des brevets

(11) EP 0 885 967 A2

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication
23.12.1998 Bulletin 1998/52

(51) Int. Cl.^{6}: **C12N 15/70**, C07K 14/245, C12N 15/67

(21) Application number 98111348.3

(22) Date of filing: 19.06.1998

(84) Designated Contracting States
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States
AL LT LV MK RO SI

(30) Priority: 20.06.1997 JP 180558/97

(83) **Declaration under Rule 28(4) EPC (expert solution)**

(71) Applicant:
HSP Research Institute, Inc.
Chuo-ku, Osaka-shi, Osaka (JP)

(72) Inventors:
- Sogo, Kazuyo
  Kyoto-shi, Kyoto (JP)
- Yanagi, Hideki
  Takarazuka-shi, Hyogo-ken (JP)
- Yura, Takashi
  Kyoto-shi, Kyoto (JP)

(74) Representative:
VOSSIUS & PARTNER
Siebertstrasse 4
81675 München (DE)

(54) **Chaperone expression plasmids**

(57) An artificial operon comprising polynucleotides encoding each of chaperones DnaK, DnaJ and GrpE; an expression plasmid carrying the operon; a cotransformant prepared by introducing the expression plasmid into *E. coli* together with a foreign protein expression vector; and a method for producing a foreign protein comprising using the cotransformant.

EP 0 885 967 A2

Description

The present invention relates to a chaperone expression plasmid. More particularly, the present invention relates to an operon comprising polynucleotides encoding each of chaperones DnaK, DnaJ and GrpE; an expression plasmid carrying the operon; a cotransformant prepared by introducing the expression plasmid into *Escherichia coli* (hereinafter simply referred to as "*E. coli*") together with an expression vector for a foreign protein; and a method for producing a foreign protein using the cotransformant.

E. coli serves ideally as a host for production of heterologous proteins at low costs and high yields, because it can easily be grown to high densities and the studies on the host-vector systems have been most advanced and many high-expression vectors have been developed. *E. coli* host-vector systems are, therefore, most widely utilized as expression systems for heterologous genes.

However, many heterologous proteins, especially eukaryotic proteins, associate with each other in cytoplasm and form biologically inactive insoluble aggregates known as "inclusion bodies" when expressed at high levels in *E. coli*. There is an advantage in formation of an inclusion body in that it is made possible to protect the expressed protein against degradation by proteases in host cells and to easily separate the inclusion body by centrifugation from the cells. In order to obtain the desired biologically active protein, however, it is necessitated that the inclusion body to be denatured and solubilized, followed by renaturation (refolding). This solubilization-renaturation process is performed on the basis of repeated trial and error for individual proteins, but often fails to achieve satisfactory recovery rates. In some cases, renaturation is not always possible. Also, not a few heterologous proteins are degraded by proteases in *E. coli* and fail to achieve high expression levels. There have not yet been found a well-established means for solving such problems of insolubilization and degradation of expression products. Attempts to mass-produce biologically active proteins in *E. coli* have not always been altogether successful.

Coexpression of chaperones and the like has been known, and a number of reports have been made. DnaK, DnaJ and GrpE are chaperones that cooperatively act in protein folding. It has been considered that the ATP bound to DnaK is first hydrolyzed upon DnaJ binding to an unfolded protein substrate, resulting in the formation of an unfolded protein-DnaJ-DnaK (ADP binding type) complex, and thereafter ADP/ATP exchange takes place by GrpE, resulting in the release of the protein substrate from the complex [Szabo, A. et al., *Proc. Natl. Acad. Sci. USA* **91**, 10345-10349 (1994)].

The *dnaK* and *dnaJ* genes are located at the same operon on the *E. coli* chromosome, while the *grpE* gene is located at a site apart from the above operon. To date, there have been reported a method of coexpression of a desired protein with DnaK alone or with both DnaK and DnaJ [Blum, P. et al., *BioTechnol.* **10**, 301-304 (1992); Perez-Perez, J. et al., *Biochem. Biophys. Res. Comm.* **210**, 524-529 (1995)]; a method of coexpression of a desired protein and DnaJ alone (Japanese Patent Laid-Open No. Hei 8-308564); a method of expression of DnaK and DnaJ, and of GrpE from respectively different plasmids [Caspers, P. et al., *Cell. Mol. Biol.* **40**, 635-644 (1994)]; and a method of independent expression of DnaK and DnaJ and of GrpE from the same plasmid using the same promoter [Stieger, M. and Caspers, P., *Immunology Methods Manual*, 39-44 (1997)]. However, these methods have the drawbacks described below.

Specifically, DnaK, DnaJ and GrpE, which act in cooperation with each other, are expected to be more effective when coexpressed, and it is very likely that their inherent chaperone function is not fully exhibited simply when DnaK alone or only DnaK and DnaJ are expressed. Also, in the method in which DnaK and DnaJ, and GrpE, are expressed from the respectively different plasmids, since it is difficult to allow a total of three plasmids, including the expression plasmid for the desired protein, to be coexisted in *E. coli*, the gene for GrpE and the gene for the desired protein are placed on a single plasmid, which in turn necessitates that the expression plasmids need to be constructed to adapt to individual desired proteins. Moreover, since the same promoter is used for expression of GrpE and the desired protein, there arises a defect in that the expression of the desired proteins cannot be increased to sufficient levels. Further, in the method in which DnaK and DnaJ, and GrpE, are independently expressed from the same plasmid using the same promoter, another problem arises in the plasmid stability because of the presence of two units of the same promoter.

It has been well known to use protease mutants of *E. coli* as hosts to reduce the degradation of foreign proteins in *E. coli*. For example, deletion mutants for Lon proteases are preferably used. In addition, there has been known a method using *rpoH* mutants to suppress Lon and Clp proteases, since the induction of their expression is controlled by σ^{32}, encoded by the *rpoH* gene (Japanese Unexamined Patent Publication No. Sho 61-501307, WO 85/03949). Also, there has been known a method for stably expressing foreign proteins using double-mutants having mutations in the *clpPX* and *lon* genes (Japanese Patent Laid-Open No. Hei 8-140671).

It should be noted, however, that σ^{32} also controls the induction of expression of chaperones, such as DnaK, DnaJ, GrpE, GroEL and GroES. GroEL and GroES are essential for the growth of *E. coli*, and *rpoH* deletion mutants cannot grow at temperatures exceeding 20°C. Therefore, missense mutations have conventionally been used for *rpoH* mutants (*htpR* mutants). It is desired, however, that the *rpoH* deletion mutants be

used to more completely suppress the induction of expression of various proteases, such as Lon protease and Clp protease.

There have been reported a large number of successful cases of solubilization of foreign proteins that otherwise remain insolubilized in *E. coli* by coexpression of the foreign protein and GroEL and GroES. Examples thereof include, for instance, tyrosine kinase [Caspers, P. et al., *Cell Mol. Biol.* **40**, 635-644 (1994); Amrein, K.E. et al., *Proc. Natl. Acad. Sci. USA* **92**, 1048-1052 (1995)]; glutamate racemase [Ashiuchi, M. et al., *J. Biochem.* **117**, 495-498 (1995)]; and dihydrofolate reductase [Dale, G.E. et al., *Protein Eng.* **7**, 925-931 (1994)]. Other reported cases include improvement of solubility of human growth hormone by coexpression of DnaK [Blum, P. et al., *Biotechnol.* **10**, 301-304 (1992)], transglutaminase solubilization by coexpression of DnaJ (Japanese Patent Laid-Open No. Hei 8-308564), and tyrosine kinase solubilization by coexpression of DnaK, DnaJ and GrpE [Caspers, P. et al., *Cell Mol. Biol.* **40**, 635-644 (1994)]. It remains very difficult, however, to predict which foreign protein and which chaperone are to be coexpressed to what extent.

Thus, the technical problem underlying the present invention was to solve the above problems in prior art. The solution to the technical problem is provided by the embodiments characterized in the claims.

Accordingly, the present invention relates to an operon comprising polynucleotides encoding chaperones which can be used for expression of a foreign protein in the cells of *E. coli* in stabilized and solubilized form.

In one embodiment, the present invention provides an expression plasmid carrying the operon.

In another embodiment, the present invention provides a cotransformant prepared by introducing the expression plasmid into *Escherichia coli* together with a foreign protein expression vector.

In still another embodiment, the present invention provides a method for producing a foreign protein using the cotransformant.

According to the present invention, a plasmid for expressing the *dnaK*, *dnaJ* and *grpE* genes joined together as a single operon under control of a single promoter has been constructed. In accordance with the present invention, the efficiency of protein folding in the DnaK/DnaJ/GrpE chaperone system is increased by expressing DnaK, DNaJ and GrpE in *E. coli*. In addition, the functions of both the DnaK/DnaJ/GrpE and GroEL/ES systems, the major chaperone systems in *E. coli*, are enhanced. Thus, the efficiency of folding of the desired protein is further increased by inserting the groESgroEL gene onto the same plasmid as described above under control of another promoter, and expressing the gene product in *E. coli* mutants including protease mutants and *rpoH* mutants. In particular, the present invention makes it possible to coexpress suitable amounts of DnaK, DnaJ and GrpE in the presence of supplemented GroEL and GroES, essential for the growth of rpoH mutants, thereby expressing the desired protein in stabilized and solubilized form.

In sum, the present invention pertains to the following:

(1) An artificial operon comprising polynucleotides encoding each of chaperones DnaK, DnaJ and GrpE;
(2) The artificial operon described in item (1) above, further comprising an inducible promoter;
(3) The artificial operon described in item (1) above, wherein the inducible promotor is selected from the group consisting of *lac*, *trp*, *araB* and *Pzt-1*;
(4) A plasmid carrying the artificial operon described in any one of items (1) to (3) above, usable for expression of DnaK, DnaJ and GrpE;
(5) The plasmid described in item (4) above, further comprising a *groE* operon ligated to an inducible promotor, the plasmid being capable for expression of DnaK, DnaJ, GrpE, GroEL and GroES;
(6) The plasmid described in item (5) above, wherein the inducible promotor ligated to a *groE* operon is selected from the group consisting of *lac*, *trp*, *araB* and *Pzt-1*;
(7) A cotransformant obtainable by introducing the plasmid described in any one of items (4) to (6) above into *E. coli* together with an expression vector for a foreign protein.
(8) The cotransformant described in item (7) above, wherein *E. coli* is a protease mutant;
(9) The cotransformant described in item (8) above, wherein the protease mutant is a *lon-clpPX* double mutant or a *lon-clpPX-hslV/U* triple mutant;
(10) The cotransformant described in item (7) above, wherein *E. coli* is a *plsX* mutant;
(11) The cotransformant described in item (7) above, wherein *E. coli* is an *rpoH* mutant;
(12) The cotransformant described in item (11) above, wherein the *rpoH* mutant is an *rpoH* deletion mutant;
(13) A method for producing a foreign protein comprising using the cotransformant described in any one of items (7) to (12) above, or item (17);
(14) A method for producing a foreign protein comprising:

(a) culturing the cotransformant described in any one of items (7) to (12) above, or item (17) under conditions that cause expression of the chaperones and the foreign protein; and
(b) recovering said foreign protein from the culture.

(15) The method described in item (13) or (14) above, wherein the cotransformant is cultured under the conditions for induction of chaperones that the expression levels of DnaK, DnaJ and GrpE,

and the expression levels of GroEL and GroES are at levels suitable for stabilization and/or solubilization of the foreign protein;

(16) A kit comprising the artificial operon described in any one of items (1) to (3) above, the plasmid described in any one of items (4) to (6) above, and/or the cotransformant described in any one of items (7) to (12) above, or item (17); and

(17) The cotransformant described in any one items (7) to (12) above or the method described in any one of items (13) to (15) above, wherein the foreign protein is selected from the group consisting of interferons, interleukins, interleukin receptors, interleukin receptor antagonists, granulocyte colony-stimulating factors, granulocyte macrophage colony-stimulating factors, macrophage colony-stimulating factors, erythropoietin, thrombopoietin, leukemia inhibitors, stem cell growth factors, tumor necrosis factors, growth hormones, proinsulin, insulin-like growth factors, fibroblast growth factors, platelet-derived growth factors, transforming growth factors, hepatocyte growth factors, bone morphogenetic factors, nerve growth factors, ciliary neurotrophic factors, brain-derived neurotrophic factors, glia cell line-derived neurotrophic factors, neurotrophine, prourokinase, tissue plasminogen activators, blood coagulation factors, protein C, glucocerebrosidase, superoxide dismutase, renin, lysozyme, P450, prochymosin, trypsin inhibitors, elastase inhibitors, lipocortin, reptin, immunoglobulins, single-chain antibodies, complement components, serum albumin, cedar pollen allergens, hypoxia-induced stress proteins, protein kinases, proto-oncogene products, transcription factors and virus-constituent proteins.

The Figures show:

Figure 1 is a schematic view showing a plasmid pG-KJE6;

Figure 2 shows results of electrophoresis of NK284 and NK287, wherein the left panel shows results of SDS-PAGE of an induction of expression of a chaperone by 1 mg/ml L-arabinose, and the right panel shows results of Western blotting showing a solubility of prourokinase (proUK), wherein S denotes a soluble fraction, and I denotes an insoluble fraction,

Figure 3 shows results of SDS-PAGE showing an induction of expression of a chaperone from pG-KJE6 in JM109, wherein the numerical figures on each lane indicate concentrations of L-arabinose (Ara) and tetracycline (Tc);

Figure 4 shows results of electrophoresis of NK241, wherein the left panel shows an induction of expression of a chaperone by various concentrations of Ara and Tc; and the right panel shows an expression of CryjII;

Figure 5 shows results of electrophoresis showing a property (solubility) of CryjII in a fraction prepared by fractionating the same samples in each lane of Figure 4 to a soluble fraction and an insoluble fraction, wherein S denotes a soluble fraction, and I denotes an insoluble fraction;

Figure 6 is a graph showing the stability of CryjII coexpressed with various chaperones, wherein the CryjII level at 0 minute is defined as 1, and a half-life of CryjII level is defined as a time period in which the remaining CryjII level is 0.5 that of the initial level;

Figure 7 shows results of electrophoresis showing expression of CryjII in various chaperone mutants, wherein MC denotes a parent strain MC4100, K^- denotes C4100 *ΔdnaK52*, J^- denotes MC4100 *ΔdnaJ259*, E^- denotes MC4100 *grpE280*, L^- denotes MC4100 *groEL44*, and S^- denotes MC4100 *groES72*, and wherein S denotes a soluble fraction, and I denotes an insoluble fraction;

Figure 8 shows results of electrophoresis, wherein the upper panel shows an induction of expression of a chaperone, and the lower panel shows the expression of CryjII, each being evaluated by various concentrations of Ara and Tc in an *rpoH* deletion mutant;

Figure 9 shows results of electrophoresis showing solubility of CryjII by fractionating the same samples of each lane of Figure 8 into a soluble fraction and an insoluble fraction, wherein S denotes a soluble fraction, and I denotes an insoluble fraction; and

Figure 10 shows results of electrophoresis, wherein the upper panel shows an induction of expression of a chaperone, and the lower panel shows the expression of ORP150, each being evaluated by various concentrations of Ara and Tc in an *rpoH* deletion mutant, wherein both ends of the lane in each panel indicate molecular weight markers, and where in the right panel S denotes a soluble fraction, and I denotes an insoluble fraction.

In the present invention, the chaperone may be any protein, as long as it is involved in protein folding. In the present invention, chaperones derived from *E. coli* are preferred. Examples of such chaperones include, for instance, DnaK, DnaJ, GrpE, GroEL, GroES, HscA/Hsc66, CbpA, HtpG, and the like. DnaK, DnaJ, GrpE, GroEL and GroES are more preferable from the viewpoint of expression of foreign proteins in a stabilized and solubilized form in *E. coli*. It is particularly preferable to use in combination with the DnaK/DnaJ/GrpE chaperone systems and the GroEL/GroES chaperone systems from the viewpoint of cooperative action of such chaperones.

The present invention provides an operon encoding the chaperone. The term "operon" used in the present invention is defined as a group of genes, each of which encodes the above-described chaperone, forming a

transcription unit under the control of a single promoter, which includes a natural or artificial operon. In the present invention, it is preferable to use an artificial operon derived from *E. coli* comprising polynucleotides encoding DnaK, DnaJ and GrpE, which is referred to as *dnaK/dnaJ/grpE* operon. Also, it is more preferable to use the *dnaK/dnaJ/grpE* operon in combination with an operon comprising polynucleotides encoding GroEL and GroES, which is referred to as a *groE* operon, GroEL and GroES being required for the growth of *E. coli*.

The *dnaK/dnaJ/grpE* operon of the present invention is capable of more efficiently exhibiting the function of chaperones expressed than known *dnaK/dnaJ* operons. Concrete examples of using prourokinase as a foreign protein are given below.

From the viewpoint of regulation of the expression level of the chaperone of the present invention, it is preferable that the promoter controlling the transcription of the above-described operon be an inducible promoter. Examples of the inducible promoter include, for instance, *lac*, *tac*, *trc*, *trp*, *araB*, *Pzt-1*, λP_L, and the like. The *lac*, *tac* and *trc* promoters can be induced by using isopropyl-1-thio-β-D-galactopyranoside (IPTG); the *trp*, *araB* and *Pzt-1* promoters can be induced by using 3-indoleacrylic acid (IAA), L-arabinose and tetracycline, respectively; and the λP_L promoter can be induced at a high temperature (42°C). Also usable is a T7 promoter, which is specifically and strongly transcribed by a T7 RNA polymerase. In the transcription by T7 RNA polymerase, induction of the above T7 RNA polymerase by using IPTG is made possible using an *E. coli* strain harboring a lysogenized λ phage carrying the T7 RNA polymerase gene located downstream of the *lac* promoter.

The above-described promoters are contained in known vectors, and they can be used after being appropriately cut out from the respective vectors with restriction endonucleases, and the like.

The plasmid of the present invention has one of the above-described operons, and expresses one of the above-described chaperones after being introduced into *E. coli*. Accordingly, plasmids carrying a *dnaK/dnaJ/grpE* operon are preferred, with greater preference given to plasmids carrying both the *dnaK/dnaJ/grpE* operon and the *groE* operon.

As described above, these plasmids preferably express chaperones of the present invention, i.e., DnaK, DnaJ and GrpE, under the control of an inducible promoter, and they more preferably express DnaK, DnaJ, GrpE, GroEL and GroES under the control of an inducible promoter.

In order to optimize the level and timing of expression of the above-described chaperones without lowering the expression level of the desired protein, it is advantageous to independently control the expression of the chaperones and that of the desired protein. It is preferred that the inducible promoter used for chaperone expression differs from the promoter used to express the desired protein. Although the promoter used to express the *dnaK/dnaJ/grpE* operon and the promoter used to express the *groE* operon may be the same, the level and timing of expression of DnaK, DnaJ and GrpE and those of expression of GroEL and GroES can be separately regulated by using different promoters. For example, a plasmid pG-KJE6 (Figure 1) is desirably used, wherein the plasmid comprises an *araB* promoter-*dnaK/dnaJ/grpE* operon and a *Pzt-1* promoter-*groE* operon.

The pG-KJE6 is a plasmid constructed on the basis of a pACYC vector [Chang, A.C.Y. and Cohen, S.N., *J. Bacteriol.* **134**, 1141-1156 (1978)]. As shown in Figure 1, the pG-KJE6 has a structure comprising a pACYC vector-derived *ori*, a Cm resistance gene, the *araB* promoter-*dnaK/dnaJ/grpE* operon, and the *Pzt-1* promoter-*groE* operon. Expression of DnaK, DnaJ and GrpE is induced by using L-arabinose, and that of GroEL and GroES is induced by using tetracycline. By adding L-arabinose end tetracycline at the same time, separately with time intervals, or at different concentrations, these two groups of chaperones can be expressed at the same time, or separately with time intervals, or at different levels as occasion demands.

Two mutually closely related plasmids cannot usually stably co-exist in the same host. This phenomenon is known as incompatibility. Any plasmid can serve as the plasmid of the present invention, as long as it has a replicon showing no incompatibility in *E. coli* with the expression vector for the desired protein. When pBR322 or another expression vector having the Col E1 replicon, for example, is used as an expression vector for the desired protein, the p15A replicon, existing in a pACYC vector, can be used for the plasmid of the present invention.

The plasmid of the present invention may further contain a selection marker gene as occasion demands in order to facilitate selection upon transformation. Examples of such selection marker genes include ampicillin resistance (Amp^r) genes, kanamycin resistance (Km^r) genes, and chloramphenicol resistance (Cm^r) genes. It is desired that the selection marker gene used be different from the selection marker gene contained in the foreign protein expression vector.

The above-described plasmids can be constructed by a method, for example, described in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Sambrook, J. et al., Cold Spring Harbor Laboratory Press, New York, 1989. The construction of the above-described plasmid pG-KJE6 is concretely described in Examples set forth below.

Methods for expression of the chaperone of the present invention using an inducible promoter, and methods for regulation of the expression levels of the chaperone of the present invention, using the above-described plasmids, are described below.

In the present invention, the term "a cotransform-

ant" refers to that obtainable by introducing one of the above-described plasmids together with a foreign protein expression vector into *E. coli*.

Any expression vector for expression of a foreign protein can serve for the present invention, as long as it causes the desired foreign protein to be expressed in *E. coli*, and as long as it does not exhibit incompatibility with the above-described plasmids. A preference is given to a vector wherein the expression of the desired foreign protein is induced by an inducible promoter.

The inducible promoters for expression of a foreign protein include the same promoters as those for expression of the chaperone described above. The expression of a chaperone of the present invention and that of the desired foreign protein can be separately induced by using an appropriate promoter different from that used to induce the expression of the chaperone of the present invention.

Also, the expression vector for expression of a foreign protein may contain a selection marker gene as occasion demands. Such selection marker genes include the same as those for expression of the chaperone described above. A double selection of cotransformants is made possible by using a selection marker gene other than that contained in the plasmid of the present invention.

E. coli strains usable in the present invention include wild strains, such as HB101, JM109, MC4100, MG1655 and W3110; and various mutants, including protease mutants, such as *lon* mutants, *clpPX* mutants, *hslV/U* mutants, *lon-clpPX* double mutants and *lon-clpPX-hslV/U* triple mutants; *plsX* mutants; *rpoH* deletion mutants; and *rpoH* missense mutants.

In the present invention, protease mutants, such as *lon* mutants, *clpPX* mutants, *hslV/U* mutants, *lon-clpPX* double mutants and *lon-clpPX-hslV/U* triple mutants; *plsX* mutants; and *rpoH* mutants, such as *rpoH* deletion mutants, can be favorably used to more stably express foreign proteins.

A preferable *lon-clpPX* double mutant is *E. coli* strain KY2263 (FERM BP-6238) derived from *E. coli* strain MC4100, prepared by introducing double deletion mutations in the *lon* and *clpPX* genes. The *E. coli* KY2263 has been deposited under accession number FERM BP-6238 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, of which the address is 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-0046, Japan; date of original deposit: February 18, 1997; and date of transfer request from the original deposit to the International Deposit under the Budapest Treaty: January 26, 1998.

Also, the term "*lon-clpPX-hslV/U* triple mutant" refers to a mutant prepared by introducing mutation in the above-described *lon-clpPX* double mutant and further in the *hslV/U* gene, which encodes HslV/U protease. A preference is given to *E. coli* strain KY2266 (FERM BP-6239) derived from *E. coli* strain MC4100, prepared by incorporating triple deletion mutations in the *lon*, *clpPX* and *hslV/U* genes. The *E. coli* KY2266 has been deposited under accession number FERM BP-6239 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, of which the address is 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-0046, Japan; date of original deposit: February 18, 1997; and date of transfer request from the original deposit to the International Deposit under the Budapest Treaty: January 26, 1998.

Also, examples of the *plsX* mutants include, for instance, a *plsX* mutant having a mutation of insertion of the tetracycline resistance gene into a position corresponding to the N-terminal region of a polypeptide encoded by *plsX* (Japanese Patent Laid-Open No. Hei 8-140671).

Examples of the *rpoH* deletion mutants include, for instance, *E. coli* MC4100 Δ*rpoH* [Zhou, Y.N. et al., *J. Bacteriol.* **170**, 3640-3649 (1988)], *E. coli* MG1655 Δ*rpoH*, and the like. In the *rpoH* deletion mutants, the expression levels of all heat shock proteins controlled by σ^{32}, including chaperones and proteases, are lowered. By sufficiently supplementing such chaperones having their expression suppressed by transformation of the *rpoH* deletion mutants with, for example, pG-KJE6, it is expected that a system of low protease contents and high chaperone contents can be provided with favorable effects for stable expression of unstable foreign proteins. Also, the *rpoH* deletion mutants are sensitive to temperature, and they usually cannot grow at temperatures exceeding 20°C. By supplementing GroEL and GroES as described above, the *rpoH* deletion mutants can grow at temperatures exceeding 20°C, and hence facilitating their handling. It is, therefore, particularly preferable to use the *rpoH* deletion mutant.

In the present invention, the foreign protein to be expressed may be any protein, as long as it is a foreign protein that is expressed in unstabilized form and/or insolubilized form in *E. coli*. Such foreign proteins include interferons, interleukins, interleukin receptors, interleukin receptor antagonists, granulocyte colony-stimulating factors, granulocyte macrophage colony-stimulating factors, macrophage colony-stimulating factors, erythropoietin, thrombopoietin, leukemia inhibitors, stem cell growth factors, tumor necrosis factors, growth hormones, proinsulin, insulin-like growth factors, fibroblast growth factors, platelet-derived growth factors, transforming growth factors, hepatocyte growth factors, bone morphogenetic factors, nerve growth factors, ciliary neurotrophic factors, brain-derived neurotrophic factors, glia cell line-derived neurotrophic factors, neurotrophine, prourokinase, tissue plasminogen activators, blood coagulation factors, protein C, glucocerebrosidase, superoxide dismutase, renin, lysozyme, P450, prochymosin, trypsin inhibitors, elastase inhibitors, lipocortin, reptin, immunoglobulins, single-chain antibodies, complement components, serum albumin,

cedar pollen allergens, hypoxia-induced stress proteins, protein kinases, proto-oncogene products, transcription factors and virus-constituent proteins.

A calcium chloride method, a rubidium chloride method, an electroporation method and other conventional methods can be employed to introduce the plasmid of the present invention together with an expression vector for a foreign protein into *E. coli*. Screening for cotransformants can be carried out using chemicals appropriate for selection marker genes. Expression of the foreign protein can, for example, be confirmed by such means as Western blotting.

The present invention further provides a method for producing a foreign protein using the above-described cotransformant. The method comprises the steps of:

(1) checking chaperone induction conditions for stabilization and/or solubilization of a foreign protein subject to expression;
(2) culturing a cotransformant to induce expression of chaperones and the foreign protein under the induction conditions checked in (1) above, and harvesting the cells; and
(3) disrupting of the harvested cells, and isolating and purifying the foreign protein using a purification method depending upon the foreign protein.

First, by taking an example of expression of prourokinase as a foreign protein, it is possible to specifically check that the chaperone function can be more effectively exhibited by coexpression of mutually cooperating DnaK, DnaJ and GrpE using the *dnaK/dnaJ/grpE* operon of the present invention, as compared to a case where only DnaK and DnaJ are expressed using a known *dnaK/dnaJ* operon.

A plasmid pAR3 (ATCC87026), the plasmid derived from the pACYC vector, and carrying a Cm resistance gene and *araC* and *araB* promoter/operator genes, is cleaved with a restriction endonuclease *Pst*I at a position downstream of the *araB* promoter, and the resulting cleaved plasmid is blunt-ended. Thereafter, an about 3 kb coding region of the *E. coli dnaK/dnaJ* operon prepared by PCR and an about 0.6 kb coding region of the *grpE* gene are inserted into appropriate sites to prepare a plasmid pKJE7 for expression of DnaK, DnaJ and GrpE from a single operon under the control of the *araB* promoter.

Next, the plasmid pKJE7 is cleaved with restriction endonucleases *Bsp*HI and *Kpn*I to remove almost the entire coding region of the *grpE* gene, and the resulting cleaved plasmid is blunt-ended. Thereafter, the resulting plasmid is self-ligated. A plasmid for expression of only DnaK and DnaJ under the control of the *araB* promoter is isolated and named as pKJ1.

Next, *E. coli* MG1655 (CGSC6300; *E. coli* Genetic Stock Center, Yale University) is transformed by the rubidium chloride method with an IPTG-inducible plasmid pUK-02pm0 [Kanemori, M. et al., *J. Bacteriol.* **176**, 5648-5653 (1994)], and one of the plasmid pKJE7 and the plasmid pKJ1 prepared above. The resulting cotransformant with pUK-02pm0 and pKJE7 and the resulting cotransformant with pUK-02pm0 and pKJ1 are isolated, and named as cotransformants NK284 and NK287, respectively.

Each of the cotransformants NK284 and NK287 prepared above are respectively cultured at 37°C in L broth supplemented with 1 mg/ml L-arabinose. When Klett Unit reaches about 40, 1 mM IPTG is added to the culture. After culturing for one hour, a portion of the culture is taken, and trichloroacetic acid is added so as to give a final concentration of 5% to precipitate the cells. Each of the precipitates is collected by centrifugation and washed with acetone. Thereafter, the washed cells are dissolved in a sample buffer for SDS-PAGE, and proteins are separated by SDS-PAGE, followed by detection of induced chaperones by CBB staining (Figure 2, left panel).

The cells of each of NK284 and NK287 recovered by centrifugation of the remaining portion of the culture mentioned above are disrupted by sonication. Thereafter, the disrupted cells are fractionated by centrifugation into a soluble fraction and an insoluble fraction to detect prourokinase in each fraction by Western blotting using an antibody against urokinase (Figure 2, right panel).

It is clear from Figure 2 that when DnaK, DnaJ and GrpE are coexpressed, almost entire prourokinase are expressed in a soluble form, whereas when only DnaK and DnaJ are coexpressed, the prourokinase expressed is only partially solubilized, the remaining being expressed in an insoluble form.

The method for producing a foreign protein using a cotransformant NK241 by using the plasmid pG-KJE6 and an expression vector for a cedar pollen allergen, such as a *Cryptomeria japonica* pollen allergen CryjII will be explained concretely hereinbelow. When expressed in *E. coli*, the CryjII is an unstable protein, its half-life is about ten minutes as determined by Western blotting of the amount of CryjII remaining in cells in which protein synthesis is blocked by addition of spectinomycin.

<u>(1) Studies on Conditions for Chaperone Induction Suitable for Stabilization and/or Solubilization of CryjII</u>

First, *E. coli* JM109 is transformed with pG-KJE6 alone, and a transformant is obtained by selection with chloramphenicol. The resulting transformant is cultured at 30°C in an L broth supplemented with 0 to 3 mg/ml L-arabinose and 0 to 150 ng/ml tetracycline. When Klett Unit reaches about 40, trichloroacetic acid is added to the culture so as to give a final concentration of 5% to precipitate the cells. Thereafter, the proteins are separated by SDS-PAGE, followed by detection of induced chaperones by Coomassie brilliant blue (CBB) staining (Figure 3). As shown in Figure 3, each of chaperones is induced which is concentration-dependent on the

chemicals used.

Next, NK241, which is an MG1655 cotransformant with pG-KJE6 and an IPTG-inducible expression vector for CryjII is cultured in the same manner as described above, except that 0 to 8 mg/ml L-arabinose and 0 to 10 ng/ml tetracycline are added. When Klett Unit reaches about 40, 1 mM IPTG is added to the culture. After culturing for two hours, a portion of the culture is taken, and trichloroacetic acid is added so as to give a final concentration of 5% to precipitate the cells. Thereafter, the proteins are separated by SDS-PAGE, followed by detection of induced chaperones by CBB staining or detection of CryjII by Western blotting (Figure 4). As shown in Figure 4, when DnaK, DnaJ and GrpE are coexpressed, or GroEL and GroES are coexpressed, or all five proteins are coexpressed, the CryjII is expressed in a high level.

Also, the cotransformant recovered by centrifugation of the remaining portion of the culture is disrupted by sonication. Thereafter, the disrupted cells are fractionated by centrifugation into a soluble fraction and an insoluble fraction to detect solubility of CryjII in each fraction by Western blotting (Figure 5). As shown in Figure 5, CryjII is expressed in an insoluble form when only DnaK, DnaJ and GrpE are coexpressed (lanes 2 to 5), while it is stabilized in a soluble form when expression of GroEL and GroES is induced at the same time in the presence of relatively low amounts of DnaK, DnaJ and GrpE expressed (lanes 6 to 9). When DnaK, DnaJ and GrpE are expressed in great excess, however, CryjII is expressed in an insoluble form even when expression of GroEL and GroES is induced at the same time (lane 10). It is, therefore, seen that when expression of GroEL and GroES is induced at the same time, CryjII insolubilization owing to overexpression of DnaK, DnaJ and GrpE is suppressed to a certain extent.

CryjII stabilization can be shown as a half-life by quantitating by Western blotting the amount of CryjII remaining in the cells in which protein synthesis is blocked by addition of spectinomycin. Under the conditions shown above, the half-life is 40 minutes or more (Figure 6).

In order to further clarify the effects of the chaperones on expression of CryjII, the above-described CryjII expression vector is introduced into each of DnaK, DnaJ, GrpE, GroEL and GroES mutants derived from MC4100 strain, and the expression and solubility of CryjII are examined in the same manner as described above (Figure 7). As shown in Figure 7, CryjII is expressed in an insoluble form in the DnaK mutant and the DnaJ mutant, while it is hardly affected in the GrpE mutant. It can be deduced that CryjII is soluble but more unstable with reduced expression levels in the GroEL mutant and the GroES mutant.

In consideration of these results, it is suggested that DnaK, DnaJ and GrpE have important effects on the CryjII folding, because the CryjII is expressed in an insoluble form when DnaK, DnaJ and GrpE are expressed in excess or in shortage.

Next, the chaperones involved in the CryjII folding are studied in further detail in the same manner as described above, using an *rpoH* deletion mutant cotransformant, NK196 (Figures 8 and 9). As shown in Figures 8 and 9, in the *rpoH* deletion mutant, the CryjII expressed is very stable but is expressed in a considerably insoluble form because of the reduced amounts of a set of chaperones and proteases (Figures 8 and 9, lane "a"). Also, regarding the CryjII solubilization, when only three of DnaK, DnaJ and GrpE, or only two of GroEL and GroES, are coexpressed, CryjII is not solubilized (Figure 9, lanes "b" and "c"). CryjII is solubilized for the first time when all five of DnaK, DnaJ, GrpE, GroEL and GroES are coexpressed (Figure 9, lane "d"). Furthermore, when the expression levels of DnaK, DnaJ and GrpE are further increased under the conditions for coexpression of the above-mentioned five proteins, re-insolubilization of CryjII takes place (Figure 9, lane "e"), yielding the experimental results which are consistent with those obtained with NK241.

When combined together, the above-described results lead to the following hypothesis: GroEL and GroES bind to CryjII to inhibit the above CryjII degradation by proteases without being much involved in CryjII folding. On the other hand, DnaK, DnaJ and GrpE are closely associated with CryjII folding, with an important role probably played by DnaJ, in particular. However, expression of DnaK, DnaJ and GrpE in excess would make CryjII in an insoluble form. Thus, in order to carry out CryjII folding efficiently, it is desired that two chaperone groups, i.e., the group of DnaK, DnaJ and GrpE, and the group of GroEL and GroES, are present in appropriate amounts. This hypothesis agrees well with the existing hypotheses of mutual cooperation of the chaperones.

It is novel to study the effects of the five chaperones of DnaK, DnaJ, GrpE, GroEL and GroES on expression of a foreign protein by coexpressing them at the same time or in groups, and their effective expression. Studying proteins, such as CryjII, of which behaviors change depending on the kinds and amounts of the chaperones coexpressed is highly interesting from the viewpoint of the understanding of chaperone action. Also, the systems in which only chaperones are overexpressed in the *rpoH* deletion mutants seem to be applicable to more efficient expression of other foreign proteins as well.

<u>(2) Cultivation of NK241, Inductive Expression of Chaperones and Foreign Proteins, and Recovery of Cells</u>

The NK241 is cultured in the same manner as in (1) above, under suitable chaperone induction conditions thus obtained for expression of CryjII in a stable and soluble form (10 ng/ml tetracycline and 1 mg/ml L-arabinose). When Klett Unit reaches about 40, 1 mM IPTG is added to the culture, and the cells are harvested after

culturing for two hours.

(3) Isolation and Purification of CryjII

After the harvested cells are disrupted, the supernatant is recovered by such as centrifugation. The resulting supernatant is subjected to conventional purification methods for proteins, such as gel filtration and various column chromatographies, to purify CryjII.

In another embodiment of the present invention, human ORP150 is produced using a cotransformant NK269 prepared by introducing into *E. coli* JM109 an expression vector pORP4 (induced with IPTG) for a human hypoxia-induced stress protein ORP150, and pG-KJE6. When human ORP150 is expressed in *E. coli* using pORP4 alone, the expressed ORP150 is mostly insoluble. Since NK269 cannot grow for unknown reasons, when L-arabinose is added to the culture at the initiation time of cultivation, NK269 is cultured to induce expression of human ORP150 in the same manner as above, except that L-arabinose and tetracycline are added when Klett Unit reaches about 40 (Figure 10). As shown in Figure 10, not less than half the human ORP150 produced appears in the soluble fraction when only GroEL and GroES are coexpressed (right panel, lane "b"), and it is mostly soluble when only three of DnaK, DnaJ and GrpE or all the above-described five are expressed at the same time (right panel, lanes "c", "d" and "e").

Human ORP150 is, therefore, produced, for example, as follows: NK269 is cultured in L broth. When Klett Unit reaches about 40, 10 ng/ml tetracycline, 10 mg/ml L-arabinose and 1 mM IPTG are added to the culture to induce expression. After 2 hours of cultivation, the cells are harvested in the same manner as above, followed by isolation and purification of ORP150.

The Examples illustrate the invention.

Unless otherwise specified, the following examples were carried out by the methods described in Sambrook, J. et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, published in 1989, *Current Protocols in Protein Science* (ed. Coligan, J.E. et al.), John Wiley and Sons, Inc., etc.

Example 1

Preparation of pKJE7

A plasmid pAR3 (ATCC87026), derived from a pACYC vector, and carrying a Cm resistance gene and *araC* and *araB* promoter/operator genes, was cleaved with a restriction endonuclease *Pst*I at a position downstream of the *araB* promoter, and the resulting cleaved plasmid was blunt-ended. Thereafter, an about 3 kb coding region of the *E. coli dnaK/dnaJ* operon prepared by PCR and an about 0.6 kb coding region of the *grpE* gene were inserted into appropriate sites to prepare a plasmid pKJE7 for expression of DnaK, DnaJ and GrpE from a single operon under the control of the *araB* promoter.

Comparative Example 1

Preparation of pKJ1

The plasmid pKJE7 prepared in Example 1 was cleaved with restriction endonucleases *Bsp*HI and *Kpn*I to remove almost the entire coding region of the *grpE* gene, and the resulting cleaved plasmid was blunt-ended. Thereafter, the resulting plasmid was self-ligated. A plasmid for expression of only DnaK and DnaJ under the control of the *araB* promoter was isolated and named as pKJ1.

Example 2

Preparation of NK284 Cotransformant

E. coli MG1655 (CGSC6300; *E. coli* Genetic Stock Center, Yale University) was transformed by the rubidium chloride method with 10 ng of a plasmid pUK-02pm0 [Kanemori, M. et al., *J. Bacteriol.* **176**, 5648-5653 (1994)], and 10 ng of the plasmid p*KJE7* prepared in Example 1, the plasmid pUK-02pm0 being capable of inducing expression of human prourokinase with IPTG. The resulting cotransformant with pUK-02pm0 and pKJE7 was isolated by selection with chloramphenicol and ampicillin, and named as a cotransformant NK284.

Comparative Example 2

Preparation of NK287 Cotransformant

Same procedures as in Example 2 were carried out except that the plasmid pKJ1 prepared in Comparative Example 1 was used in place of the plasmid pKJE7 in Example 2. A cotransformant with pUK-02pm0 and pKJ1 was isolated and named as a cotransformant NK287.

Test Example 1

Expression of Prourokinase Using NK284 and NK287

The cotransformant NK284 prepared in Example 2 and the cotransformant NK287 prepared in Comparative Example 2 were respectively cultured at 37°C in L broth supplemented with 1 mg/ml L-arabinose (manufactured by Wako Pure Chemical Industries). When Klett Unit reached about 40, 1 mM IPTG (manufactured by Wako Pure Chemical Industries) was added to the culture. After culturing for one hour, a portion of the culture was taken, and trichloroacetic acid was added so as to give a final concentration of 5% to precipitate the cells. Each of the precipitates was collected by centrifu-

gation and washed with acetone. Thereafter, the washed cells were dissolved in a sample buffer for SDS-PAGE, and proteins were separated by SDS-PAGE, followed by detection of induced chaperones by CBB staining (Figure 2, left panel).

The cells of each of NK284 and NK287 recovered by centrifugation of the remaining portion of the culture mentioned above were disrupted by sonication. Thereafter, the disrupted cells were fractionated by centrifugation into a soluble fraction and an insoluble fraction to detect prourokinase in each fraction by Western blotting using an antibody against urokinase (manufactured by SANBIO BV) (Figure 2, right panel).

Example 3

Preparation of pG-KJE6

The luciferase gene, located downstream of the *Pzt-1* promoter in a plasmid pUHE2Pzt-1 (made available by Dr. H. Bujard of Heidelberg University, Germany), the plasmid pUHE2Pzt-1 carrying the *Pzt-1* promoter, was cut out with restriction endonucleases *Kpn*I and *Xba*I and ligated to the *E. coli groE* operon lacking its own promoter region, the *E. coli groE* operon being prepared by digesting pKV1561 [Kanemori, M. et al., *J. Bacteriol.* **176**, 4235-4242 (1994)] with a restriction endonuclease *Xho*I, to prepare a plasmid pGro8 for expression of GroEL and GroES under the control of the *Pzt-1* promoter. Subsequently, the tetracycline repressor (*tetR*) gene of about 800 bp was prepared from an *E. coli* strain having a transposon *Tn10* by PCR, and the resulting gene was inserted into the *Aat*I site upstream of the *Pzt-1* promoter of pGro8, to give pGro10R.

Next, the resulting pGro10R was cleaved with restriction endonucleases *Sac*I and *Avr*II to prepare a fragment containing *tetR-Pzt-1-groESgroEL*. The resulting fragment was then blunt-ended and inserted into the *Xmn*I site of the pKJE7 prepared in Example 1, to prepare a plasmid pG-KJE6 for expression of DnaK, DnaJ and GrpE under the control of the *araB* promoter and for expression of GroEL and GroES under the control of *Pzt-1*.

Example 4

Induction Expression of Chaperone from pG-KJE6 in *E. coli* JM109

E. coli JM109 (TaKaRa Competent Cell, manufactured by Takara Shuzo Co., Ltd.) was transformed by the rubidium chloride method with 10 ng of the pG-KJE6 prepared in Example 3. The transformants resulting from selection with chloramphenicol were cultured at 30°C in L broth supplemented with 0 to 3 mg/ml L-arabinose (manufactured by Wako Pure Chemical Industries) and 0 to 150 ng/ml tetracycline (manufactured by Nacalai Tesque). When Klett Unit reached about 40, trichloroacetic acid was added to the culture so as to give a final concentration of 5% to precipitate the cells. Each of the precipitates was collected by centrifugation and washed with acetone. Thereafter, the washed cells were dissolved in a sample buffer for SDS-PAGE, and proteins were separated by SDS-PAGE, followed by detection of induced chaperones by CBB staining (Figure 3).

Example 5

Preparation of NK241 cotransformant

A region encoding mature CryjII protein (Arg^{46}-Ser^{433}) of a *Cryptomeria jabonica* pollen allergen CryjII cDNA [Namba, M. et al., *FEBS Lett.* **353**, 124-128 (1994)] was inserted into the *Eco*RI-*Pst*I site of the IPTG-inducible expression plasmid pKK223-3 for *E. coli* (manufactured by Pharmacia Biotech), to prepare pKCJ2. Subsequently, the *lacI^q* gene prepared from pMJR1560 (manufactured by Amersham) was inserted into the *Bam*HI site of pKCJ2 to give pKCJ2I.

E. coli MG1655 (CGSC6300; *E. coli* Genetic Stock Center, Yale University) was transformed by the rubidium chloride method with 10 ng of the pG-KJE6 prepared in Example 3 and 10 ng of the CryjII expression vector pKCJ2I described above. The resulting cotransformants were isolated by selection with chloramphenicol and ampicillin and named as cotransformant NK241.

Example 6

Expression of CryjII Using NK241

NK241 prepared in Example 5 was cultured in the same manner as in Example 4, except that 0 to 8 mg/ml L-arabinose and 0 to 10 ng/ml tetracycline were added. When Klett Unit reached about 40, 1 mM IPTG was added to the culture. After culturing for two hours, a portion of the culture was taken, and trichloroacetic acid was added so as to give a final concentration of 5% to precipitate the cells. The precipitates were collected by centrifugation and washed with acetone. Thereafter, the washed cells were dissolved in a sample buffer for SDS-PAGE, and proteins were separated by SDS-PAGE, followed by detection of induced chaperones by CBB staining. Furthermore, CryjII was detected by Western blotting using a monoclonal antibody N-26 raised against CryjII [Sawatani et al., *Allergy*, **43**, 467-473 (1984)] (Figure 4).

Also, the NK241 cells recovered by centrifugation of the remaining portion of the culture were disrupted by sonication. Thereafter, the disrupted cells were fractionated by centrifugation into a soluble fraction and an insoluble fraction to detect CryjII in each fraction by Western blotting in the same manner as above (Figure 5).

Example 7

Stability of CryjII Expressed in NK241

NK241 prepared in Example 5 was cultured in the same manner as in Example 4, except that 20 ng/ml tetracycline, or 8 mg/ml L-arabinose or both 20 ng/ml tetracycline and 8 mg/ml L-arabinose were added. When Klett Unit reached about 40, 1 mM IPTG was added to the culture. After culturing for two hours, expression of CryjII was induced. Spectinomycin (manufactured by Sigma) was then added so as to give a final concentration of 500 μg/ml to stop protein synthesis. Thereafter, samples were taken at given intervals, and cells were collected. A total protein of each of the cells was separated by SDS-PAGE, and Western blotting was then carried out using a monoclonal antibody N-26 raised against CryjII. The resulting Western blotting image was captured with a scanner, and the band intensity was assayed using an analytical software Intelligent Quantifier (manufactured by Nihon Bioimage) (Figure 6).

Example 8

Expression of CryjII in Various Chaperone Mutants

E. coli MC4100 Δ*dnaK52* [Nagai, H. et al., *Proc. Natl. Acad. Sci. USA* **91**, 10280-10284 (1994)] was used as a DnaK mutant, *E. coli* MC4100 Δ*dnaJ259* [Ishiai, M. et al., *J. Bacteriol.* **174**, 5597-5603 (1992)] as a DnaJ mutant, *E. coli* MC4100 *grpE280* [Ishiai, M. et al., *J. Bacteriol.* **174**, 5597-5603 (1992)] as a GrpE mutant, *E. coli* MC4100 *groEL44* [Tilly, K. and Georgopoulos, C., *J. Bacteriol.* **149**, 1082-1088 (1982)] as a GroEL mutant, and *E. coli* MC4100 *groES72* [Tilly, K. and Georgopoulos, C., *J. Bacteriol.* **149**, 1082-1088 (1982)] as a GroES mutant. According to the method described in Example 5, 10 ng of the CryjII expression vector was introduced into each of these mutants, and the expression and solubility of CryjII in each mutant were examined in the same manner as in Example 6 (Figure 7).

Example 9

Expression of CryjII in *rpoH* Deletion Mutant

The Δ*rpoH::kan* gene of *E. coli* MC4100 Δ*rpoH* [Zhou, Y.N. et al., *J. Bacteriol.* **170**, 3640-3649 (1988)] was transferred into *E. coli* MG1655 by transduction using T4 phage. A strain having Δ*rpoH::kan* transferred thereinto was selected using kanamycin resistance as an index. Having confirmed that the strain grew at 20°C, while it could not grow at 30°C, 37°C or 42°C, *E. coli* MG1655 Δ*rpoH* strain, NK161, was obtained.

Same procedures as in Example 5 were carried out, except that *E. coli* MG1655 Δ*rpoH* strain, NK161, described above was used in place of *E. coli* MG1655 in Example 5, to give an *rpoH* deletion mutant cotransformant NK196. The expression and solubility of CryjII were examined for the resulting deletion mutant cotransformant in the same manner as in Example 6 (Figures 8 and 9).

Example 10

Preparation of NK269 Cotransformant

A region encoding mature ORP150 protein (Leu^{33}-Leu^{999}) of a human ORP150 cDNA [Ikeda, J. et al., *Biochem. Biophys. Res. Comm.* 230, 94-99 (1997)] was inserted into the *Nco*I site of the IPTG-inducible expression plasmid pTrc99A for *E. coli* (manufactured by Pharmacia Biotech) to prepare pORP4. *E. coli* JM109 was transformed with 10 ng of resulting pORP4 and 10 ng of pG-KJE6 prepared in Example 3 according to the method described in Example 4, to give a cotransformant NK269.

Example 11

Expression of Human ORP150 Using NK269

Since NK269 prepared in Example 10 could not grow when L-arabinose was added to the culture at the initiation time of cultivation, NK269 was cultured to induce expression of human ORP150 in the same manner as Example 6, except that L-arabinose and tetracycline were added when Klett Unit reaches about 40 (Figure 10).

According to the present invention, there can be provided an operon comprising polynucleotides encoding chaperones which can be used for expressing a foreign protein in *E. coli* cells in a stabilized and solubilized form, a plasmid for expression having the operon, a cotransformant prepared by introducing the plasmid into *E. coli* together with an expression vector for a foreign protein, and a method for producing a foreign protein using the cotransformant. According to the present invention, an efficient production of a foreign protein in *E. coli* by means of genetic engineering techniques is made possible.

Claims

1. An artifcial operon comprising polynucleotides encoding each of chaperones DnaK, DnaJ and GrpE.

2. The artificial operon according to claim 1, further comprising an inducible promoter.

3. A plasmid carrying the artificial operon according to claims 1 or 2, usable for expression of DnaK, DnaJ and GrpE.

4. The plasmid according to claim 3, further comprising a *groE* operon ligated to an inducible promoter, the plasmid being capable for expression of DnaK, DnaJ, GrpE, GroEL and GroES.

5. The artificial operon according to claim 2 or the plasmid according to claim 4, wherein said inducible promoter is selected from the group consisting of *lac*, *trp*, *araB* and *Pzt-1*.

6. A cotransformant obtainable by introducing the plasmid according to any one of claims 3 to 5 into *E. coli* together with an expression vector for a foreign protein.

7. The cotransformant according to claim 6, wherein *E. coli* is a protease mutant, a *plsX* mutant, or an *rpoH* mutant.

8. The cotransformant according to claim 7, wherein said protease mutant is a *lon-clpPX* double mutant or a *lon-clpPX-hslV/U* triple mutant.

9. The cotransformant according to claim 7, wherein said *rpoH* mutant is an *rpoH* deletion mutant.

10. A method for producing a foreign protein comprising using the cotransformant according to any one of claims 6 to 9.

11. A method for producing a foreign protein comprising:

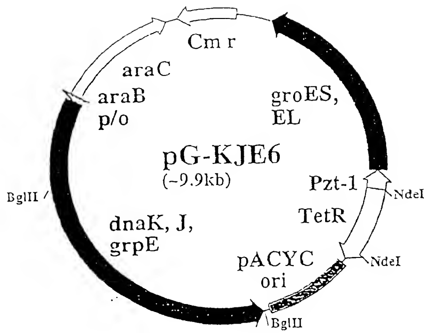
(a) culturing the cotransformant according to any one of claims 6 to 9 under conditions that cause expression of the chaperones and the foreign protein; and
(b) recovering said foreign protein from the culture.

12. The method according to claim 10 or 11, wherein the cotransformant is cultured under the conditions for induction of chaperones that the expression levels of DnaK, DnaJ and GrpE, and the expression levels of GroEL and GroES are at levels suitable for stabilization and/or solubilization of the foreign protein.

13. A kit comprising:

(a) the artificial operon according to claim 1 or 2;
(b) the plasmid according to any one of claims 3 to 5; and/or
(c) the cotransformant according to any one of claims 6 to 9.

14. The cotransformant according to any one of claims 6 to 9 or the method according to any one of claims 10 to 12, wherein said foreign protein is selected from the group consisting of interferons, interleukins, interleukin receptors, interleukin receptor antagonists, granulocyte colony-stimulating factors, granulocyte macrophage colony-stimulating factors, macrophage colony-stimulating factors, erythropoietin, thrombopoietin, leukemia inhibitors, stem cell growth factors, tumor necrosis factors, growth hormones, proinsulin, insulin-like growth factors, fibroblast growth factors, platelet-derived growth factors, transforming growth factors, hepatocyte growth factors, bone morphogenetic factors, nerve growth factors, ciliary neurotrophic factors, brain-derived neurotrophic factors, glia cell line-derived neurotrophic factors, neurotrophine, prourokinase, tissue plasminogen activators, blood coagulation factors, protein C, glucocerebrosidase, superoxide dismutase, renin, lysozyme, P450, prochymosin, trypsin inhibitors, elastase inhibitors, lipocortin, reptin, immunoglobulins, single-chain antibodies, complement components, serum albumin, cedar pollen allergens, hypoxia-induced stress proteins, protein kinases, proto-oncogene products, transcription factors and virus-constituent proteins.


Cm r
araC
araB
p/o
groES,
EL
pG-KJE6
(~9.9kb)
Pzt-1
NdeI
BglII
TetR
dnaK, J,
grpE
pACYC
ori
NdeI
BglII

F I G. 1

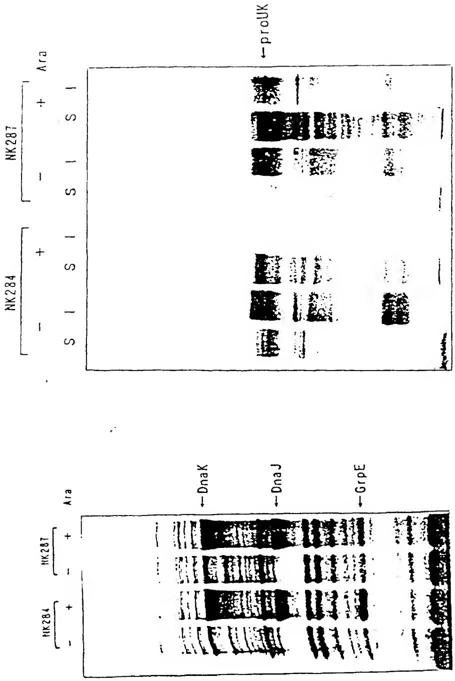


FIG. 2

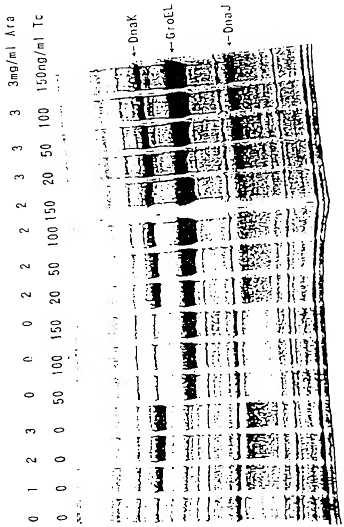

3mg/ml Ara
150ng/ml Tc
0 1 2 3 0 0 0 2 2 2 2 3 3 3 3
0 0 0 0 50 100 150 20 50 100 150 20 50 100 150
DnaK
GroEL
DnaJ

FIG. 3

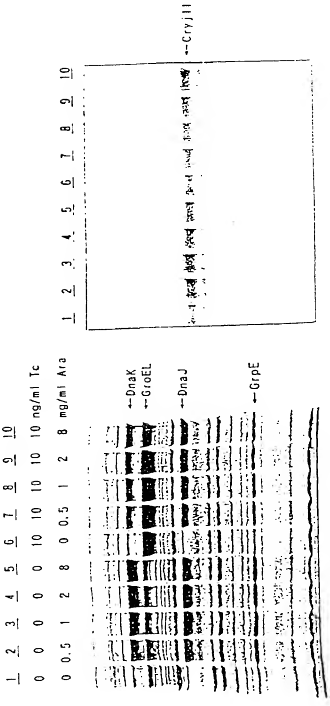

1 2 3 4 5 6 7 8 9 10
0 0 0 0 0 10 10 10 10 10 ng/ml Tc
0 0.5 1 2 8 0 0.5 1 2 8 mg/ml Ara
←DnaK
←GroEL
←DnaJ
←GrpE
1 2 3 4 5 6 7 8 9 10
←CryjII

FIG. 4

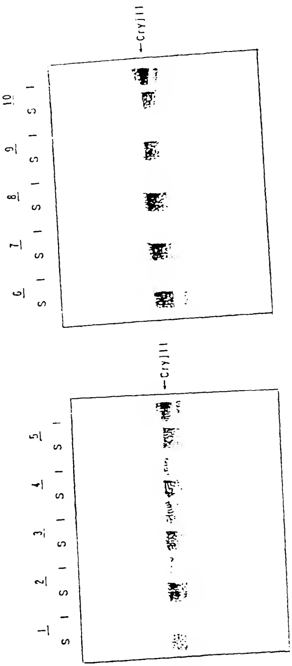


FIG. 5

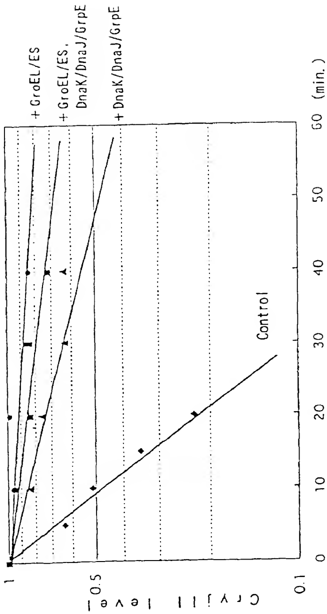

+ GroEL/ES
+ GroEL/ES,
DnaK/DnaJ/GrpE
+ DnaK/DnaJ/GrpE
Control
CryIII level
1
0.5
0.1
0
10
20
30
40
50
60 (min.)

FIG. 6

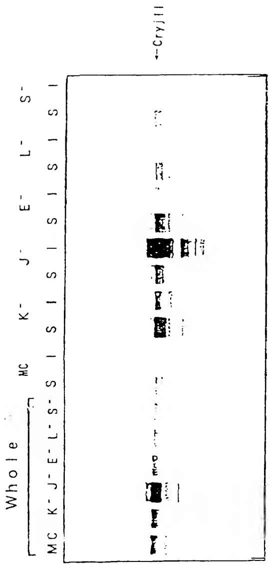
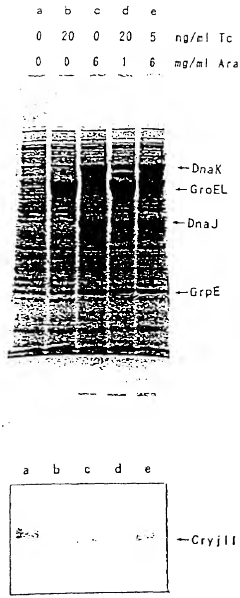
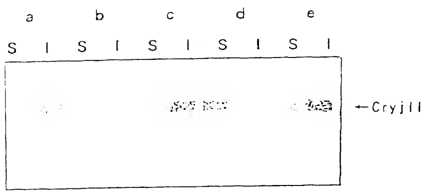


FIG. 7


a b c d e
0 20 0 20 5 ng/ml Tc
0 0 6 1 6 mg/ml Ara
DnaK
GroEL
DnaJ
GrpE
a b c d e
CryjII

F I G. 8



F I G. 9

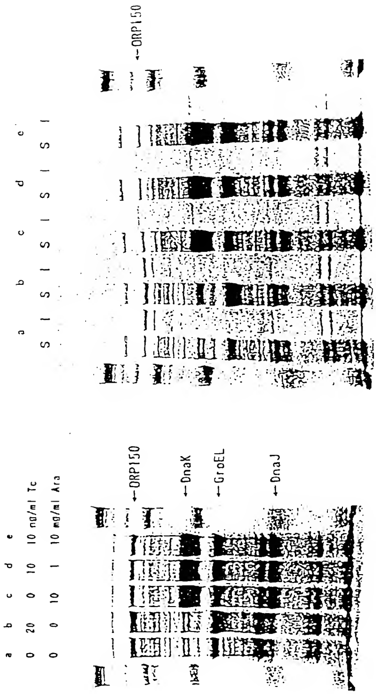

a b c d e
S I S I S I S I S I
←ORP150
a b c d e
0 20 0 10 10 ng/ml Tc
0 0 10 1 10 mg/ml Ara
←ORP150
←DnaK
←GroEL
←DnaJ

FIG. 10

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 885 967 A3

(12) EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
21.06.2000 Bulletin 2000/25

(51) Int. Cl.[7]: **C12N 15/70**, C07K 14/245, C12N 15/67

(43) Date of publication A2:
23.12.1998 Bulletin 1998/52

(21) Application number: 98111348.3

(22) Date of filing: 19.06.1998

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 20.06.1997 JP 18055897

(83) **Declaration under Rule 28(4) EPC (expert solution)**

(71) Applicant:
HSP Research Institute, Inc.
Chuo-ku, Osaka-shi, Osaka (JP)

(72) Inventors:
- **Sogo, Kazuyo**
  Kyoto-shi, Kyoto (JP)
- **Yanagi, Hideki**
  Takarazuka-shi, Hyogo-ken (JP)
- **Yura, Takashi**
  Kyoto-shi, Kyoto (JP)

(74) Representative:
VOSSIUS & PARTNER
Siebertstrasse 4
81675 München (DE)

(54) **Chaperone expression plasmids**

(57) An artificial operon comprising polynucleotides encoding each of chaperones DnaK, DnaJ and GrpE; an expression plasmid carrying the operon; a cotransformant prepared by introducing the expression plasmid into *E. coli* together with a foreign protein expression vector; and a method for producing a foreign protein comprising using the cotransformant.

EP 0 885 967 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 98 11 1348

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WU, S.C. ET AL.: "Enhanced Secretory Production of a Single-Chain Antibody Fragment by an Engineered Bacillus subtilis Strain Which Overproduces Various Molecular Chaperones" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 15, no. 23, 1996, page 505 XP000891852 abstract number: H-128 * the whole document * ---	1	C12N15/70 C07K14/245 C12N15/67
P,X	NISHIHARA, K. ET AL.: "Chaperone Coexpression Plasmids: Differential and Synergistic Roles of DnaK-DnaJ-GrpE and GroEL-GroES in Assisting Folding of an Allergen of Japanese Cedar Pollen, Cryj2, in Escherichia coli" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 64, no. 5, May 1998 (1998-05), pages 1694-1699, XP002135426 * the whole document * ---	1-7, 9-12,14	
D,A	STIEGER, M. & CASPERS, P.: "The production of soluble recombinant proteins in E. coli assisted by molecular chaperones" October 1996 (1996-10) , ACADEMIC PRESS LTD XP002135427 chapter 1.4 * page 40 - page 44 * -----	1-14	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N C07K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	11 April 2000	Fuchs, U

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)